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14. ABSTRACT Over the first 6 months of this award, we have focused on building a strong team, clinical collaborations, patient enrollment and samples acquisition. Our team has tested and optimized a number of protocols to process samples, generate antigen-presenting cells, and expand T cells for testing against tumor lysates from the Spellman group. This process is nearly complete, and we are positioned to work through the entire process as outlined in the SOW. We anticipate tumor-reactive T cell clones from patients will be generated starting in Year 2, which will lead downstream to antigen discovery.					
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Annual Progress Report 4/1/12-10/15/12 (partial year)

DoD Multi-Team Award

Enhancing the Breadth and Efficacy of Therapeutic Vaccines for Breast Cancer

Peter P. Lee, M.D.

City of Hope Cancer Center, Duarte, CA

INTRODUCTION

The immune response offers exquisite specificity and the potential to target tumor cells without harming normal cells. Inducing an effective immune response via therapeutic vaccines for cancer had been a promising but elusive goal for years. For breast cancer (BC), vaccine efforts have largely focused on eliciting immune responses to HER2. While HER2 is generally assumed to be a good antigen in HER2-overexpressing tumors, HER2-specific T cells exist at very low levels (less than 0.1%) in peripheral blood of such patients (Inokuma, dela Rosa et al. 2007). Hierarchy of the T cell repertoire and negative selection can shape immune responses in ways not readily predictable from protein expression levels alone. Thus, targeting a single antigen such as HER2 in breast cancer is likely to be insufficient - instead we need a repertoire of multiple immunologically validated T cell antigens present in breast cancers that can be deployed in a patient-specific manner. Research has focused on stimulating T cells using many pathways including the T cell antigen receptor (TCR), via co-stimulatory pathways, and manipulating the tumor environment. To optimally activate pre-existing anti-tumor T cells in BC patients, the antigens to which these T cells target must be determined. It is now recognized that invasive ductal carcinoma of the breast is a heterogeneous disease consisting of several major molecularly defined subtypes, including Luminal A, Luminal B, HER2+, and Basal (also known as 'triple-negative', and includes the 'claudin-low' subset). These subtypes have distinct clinical, genomic and proteomic features, and it is becoming clear that there are differences between BC subtype and response to specific therapeutic agent. These results, combined with the differences in gene expression that define the distinct subtypes, make it likely that each BC subtype elicits immune responses via distinct sets of antigens, and may evade T cell-mediated killing by distinct mechanisms. Based on these newly discovered features of BC and the host immune response, this project seeks to develop a robust portfolio of immunologically validated antigens for the major BC subtypes, including those that target breast cancer stem cells, that can be used in a patient-specific manner for therapeutic vaccination, as well as to identify drugs that can synergize with these novel immunotherapies. The ultimate goal is to match these antigens and drugs to each patient's tumor subtype, thereby treating each patient with the most potent combinations and opening the door to personalized immunotherapy for breast cancer. This multi-team project will use a number of novel immunological approaches to look for evidence of BC subtype specific tumor-reactive T cells within the tumor and/or tumor-draining lymph nodes (TDLNs) including isolating, expanding and cloning tumor-reactive T cells which will culminate in a robust portfolio of immunologically validated antigens for the major breast cancer subtypes, including those that target breast cancer stem cells. We seek to expand and enhance the function of these pre-existing anti-tumor T cells in patients by discovering their natural antigens, and identifying mimotopes that broadly activate them with even higher potency. Furthermore, we will enhance the efficacy of these T cells by identifying existing drugs that promote cancer cell apoptosis but have little or no negative effect on T cells. All of these antigens and agents can be matched to each patient's tumor subtype and other molecular characteristics, thereby opening the door to personalized immunotherapy.

BODY:

This award to our team did not activate until April 2012 due to my move from Stanford to the CoH (CoH). As such, this report only covers an actual working period of 6 months. Since this award activated, I have recruited a strong team and collaborators at CoH to carry out this project. This includes two PhD postdoctoral fellows and two research associates. In addition, I formed collaborations with breast cancer surgeons, medical oncologists, and pathologists at CoH who will refer patients to our study and help us acquire tissue and blood samples. CoH breast cancer surgeon Dr. John Yim will be a key clinical collaborator on this project. We have spent several months working closely with the CoH IRB office on a human subjects protocol which was approved in May by the CoH IRB (protocol ID 11273)

and then in July by the DoD HSRRB (A-16969). This has enabled us to work closely with our surgery and pathology colleagues to develop an efficient system of identifying, recruiting, and consenting subjects, and to obtain samples from the operating room to pathology and to our laboratory. So far, we have tested a number of protocols to maximize recovery of immune cells from tumor and lymph node specimens. Below is a summary of our progress in relation to our proposed SOW tasks:

1. Generate reagents and identify conditions for experiments to follow: months 1-40, Lee, Slansky, and Spellman
2. Enroll 100 patients with all major breast cancer subtypes from the City of Hope Cancer Center (CoH): months 1-36, Lee
3. Process patient samples (blood, TDLNs, tumor): months 1-38, Lee and Spellman
4. Identify and isolate anti-tumor T cells from TDLNs and tumor samples: months 1-40, Lee, Slansky, and Spellman
5. Generation and initial analysis of T cell clones: months 1-40, Lee, Slansky, and Spellman
6. Determine antigens as subtype-specific, stem-specific, or shared (Aim 4a): months 12- 40, Lee, Slansky and Spellman
7. Identify antigens that target breast cancer stem cells (Aim 3b): months 12-40, Lee, Slansky and Spellman

Sample Acquisition

Our progress thus far has focused on task 1. As such, patient samples that have been acquired have been used for testing and optimizing progressing conditions. No subject has been officially enrolled into this study since July 2012, when we received full human subjects approvals. However, we have received de-identified tissue specimens (not traceable back to the patient) consisting of blood, lymph node, and/or tumor from 32 individuals which have been used to optimize various assays. All subjects were diagnosed without a history of any immune disorder prior to breast cancer diagnosis and had their surgical treatments at CoH. Written informed consent had been obtained from all participants according to CoH and HIPAA regulations through a tissue banking protocol. Patients' heparinized peripheral blood samples, breast tumor tissue, tumor draining lymph node (TDLN: non-sentinel lymph node and/or sentinel lymph node) were collected and have been utilized for optimization purposes.

The Denver and CoH teams have designed a flow chart for the distribution of patient cells between labs (Figures 1-3). The flow charts represent an ideal situation, which are heavily dependent on the sample sizes we receive and success of upstream protocols.

Process of Patient Samples

HLA typing

We have arranged to have patients' PBMC DNA HLA typed through the Histocompatibility lab here at CoH. CoH's Histocompatibility Laboratory is fully accredited by The American Society of Histocompatibility and Immunogenetics (ASHI), College of American Pathologists (CAP), and Clinical Laboratory Improvement Amendments (CLIA 88). They will carry out the typing using the sequence-specific oligonucleotide probe (SSOP) method. The SSOP method allows the HLA lab to define the HLA type of our patient subjects to the allele level (so called '4 digits'). Initially we will ask for the allele level typing of subjects for only HLA-A2 and HLA-DR, but information on other alleles is available at a later date if desired.

EBV transformation to generate B-LCL

The generation of antigen presenting cells (APCs) for use in presenting tumor antigen to patient T cells is a critical component of this project. We have identified two possible sources of APCs: Epstein Barr Virus (EBV) transformed B cells and monocyte-derived dendritic cells (mDCs), both of which will be generated from patient's autologous PBMCs (Figure 4). EBV transformed B cells become an immortal cell line capable of relatively easy expansion and successful cryopreservation, thus being a plentiful source of autologous APCs.

However, the transformation process is slow, taking at least 4 weeks, and EBV transformed B cells lack the degree of costimulation molecules that DCs have. DCs are potent professional APCs. However, generation of mDCs is dependent on the starting number of monocytes, and they generally do not expand in culture and are less resilient to cryopreservation. Due to the benefits and drawbacks of each APC type, we have decided to attempt to generate both for each patient enrolled into this study. DCs will be the preferred APC for presentation of tumor antigen, but EBV transformed B cells will be available if the DC approach is unsuccessful.

For the generation of EBV transformed B cells we have acquired the marmoset B95-8 cell line, which releases high titers of transforming EBV. We have expanded the B95-8 cells in culture for several weeks to a high cell number. Cells were then lysed by a freeze-thaw method using a dry ice ethanol water bath and a 37°C water bath. The EBV containing supernatant produced by this process is capable of specifically transforming B cells as the virus gains entry into the B cell via the CD21 molecule only found on B cells. To date we have successfully transformed at least three PBMC populations from healthy donors. We are currently working on increasing the speed and consistency of transformation. To do so we plan on isolating B cells from PBMCs using Life Sciences Dynall Flow Comp CD19 kit, and transforming the isolated cells. Using a high percentage, concentrated starting population of EBV targets should result in better transformation rates.

For the generation of mDCs, we have identified two methods for isolation of monocytes from PBMCs. The first method is to simply plate PBMCs onto a tissue culture dish and allow them to incubate at 37°C for 2-24hrs. During this time the monocyte population will adhere to the bottom of the dish, while the other PMBC cells will not. After the non-adherent cells are removed, the remaining monocyte population can be cultured for DC generation. The second method is magnetic isolation of monocytes using CD14 antibodies. To date, we have used Life Sciences Dynall CD14 Dynabead positive isolation kit, which produces over 95% purity in the monocytes population. Unfortunately, this kit contains nonreleasable beads that are extremely autofluorescent, making purity assessment by flow cytometry difficult, and likely to be phagocytized by the cells during culture. We have recently tested the Life Sciences Dynall CD14 Flowcomp isolation kit, which features the same isolation technology as the previously mentioned kit, but allows for the release of beads from positively selected monocytes.

Once monocytes have been isolated they can be cultured with Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (O'Neill and Bhardwaj 2005). After 6-7 days of culture, immature DCs are readily apparent by viewing under the microscope and appear as both CD11c and CD1c positive by flow cytometry. For proper antigen presentation function, these DCs are then collected and matured for 24 - 48 hrs with several factors. Currently we are comparing three maturation factors for the best induction of T cell proliferation and activation; lipopolysaccharide (LPS), transforming growth factor beta (TGFβ), and monocyte conditioned media (MCM), which is composed of three proinflammatory cytokines (IL-1β, IL-6, and TNFα) and prostaglandin E2 (PGE2). Comparison of these maturation factors will be done by examining various maturation markers on DC surfaces using flow cytometry. These markers include upregulation of CD83, CD86, and class I and II HLA molecules. Functional testing of these DCs will be done by pulsing them with CMV peptide pools (Miltenyi Biotec) during their maturation, followed by co-culturing these DCs with autologous T cells for 3-5 days. Following co-culture T cells will be examined for proliferation (CFSE staining) and activation markers CD107, CD154, CD69. The maturation method that consistently results in the strongest T cell proliferation, minus background proliferation, will be chosen as our preferred maturation method.

As of yet, we have not tested EBV transformed B cells for their APC function. It does not appear from a literature search that these cells require maturation for effective presentation of antigen. It is also possible that the DCs or transformed B cells may require an osmotic stress protocol which would allow antigen to be taken up into the endogenous antigen pathway so that it is presented on class I molecules for presentation to CD8 cells.

Generating single cell suspensions from tumor and TDLN samples

We are using a modified version of tissue dissociation protocols our lab has used in past projects. The general protocol involves mincing the tissue with small scalpel blades in a small petri dish. If the tissue is tumor involved, such as breast tumor tissue or LN tumor + tissue, the mincing is followed by digestion with collagenase and DNase for 1-2 hours, depending on how well the tissue dissociates during incubation at 37°C. Previously our lab has used Worthington Type III Collagenase for tumor involved tissue digestion. For this project, we have switched to using Roche Liberase TM Collagenase. Liberase is a more uniform product of purified enzyme blends with reduced endotoxins and other contaminants than regular collagenase products. This results in increased cell viability and cell yield, in addition to consistent performance from lot-to-lot.

Isolating and expanding T cells

Once our tissue has been processed and digested into a single cell suspension, T cells are isolated by using antibody based magnetic separation. We will be using Life Sciences Dynall FlowComp CD3, CD4, and CD8 isolation kits, which allow the magnetic beads to be released from the antibody following isolation of the desired cell type. CD3 isolation with this method has resulted in greater than 95% purity of CD3+ cells (Figure 5). CD8 and CD4 isolations are done one after the other to isolate the separate populations when needed. We have recently determined that when isolating from tumor tissue it is important to isolate CD8 T cells first, followed by CD4 T cells. This method allows for greater purity in the CD4 population. Currently we are working on improving the protocols, based on conversations with Dynall technical support, to increase our recovery of desired T cell populations. To date, we have adjusted incubation times and reagent volumes to improve the recovery, for which we are hoping to soon achieve close to 100%. Once T cells are isolated, they will enter a short in vitro expansion protocol. This protocol is based on using Life Sciences Dynall CD3/CD28 activation beads. Other stimulation agents being tested were PHA, plate bound CD3 antibodies, and other activation beads. We found that PHA, which is a mitogen, would be potentially too strong of a stimulant and cause a significant reduction in desired cell viability. CD3/CD28 beads, however, naturally reflect APC stimulation and generates a potent, yet viable, proliferative response from T cells. Dynall CD3/CD28 beads have a diameter of 4.5µm, as compared to other company beads that have 50nm diameters. Theoretically this larger size would more accurately reflect an APC size and APC-T cell synapse formation.

Our testing showed that Dynall CD3/CD28 beads consistently resulted in 20-200 fold expansion of healthy donor T cells after 7 days (Figures 6 and 7). We did further experiments to determine if feeder cells were necessary for successful T cell expansion and found that it was not necessary. Although their presence slightly enhanced the fold increase of T cells, we concluded that the slight benefit did not warrant the more uniform protocol of using just CD3/CD28 activation beads. We also examined the levels of IL-2 needed in these cultures for successful activation, and similarly compared the use of IL-2 to IL-15. For IL-2 we tested initial concentrations between 6,000 and 50 IU/mL. Cells were fed with additional IL-2 on Days 3 and 5 with half the initial dose. For IL-15 we tested initial concentrations between 200 ng/mL and 50 ng/mL, in addition to combination dosing with IL-2. We saw no differences in T cell expansion fold either between IL-2 and IL-15 or within the selected doses. We believe this is due to the potency of the CD3/CD28 beads in stimulating T cell autocrine IL-2 production. Our conclusion was to use an initial concentration of 200 IU/mL of IL-2, since IL-2 at that level has been shown to reverse T cell anergy (Bendixsen and Rekvig 2004). Although IL-15 showed no greater increase in expansion than IL-2, we may reexamine its use in the future, as IL-15 has been shown to be more potent in expanding tumor infiltrating T cells (TILs).

Our immediate concerns for the T cell expansion protocol were to remove the TILs from an adverse tumor environment and present them with factors to potentially reverse any type of dysfunction that may have been induced. We believe that we can achieve this with the use of IL-2 and CD3/CD28 beads. Following identification of our expansion protocol on healthy donor T cells, we began testing on patient TILs and LN T cells. We have successfully expanded 2 patient TIL T cell populations out of an attempted 7, 5 out of 5 SLN Tumor – populations, and 1 out of 2 SLN Tumor + populations. We expect the rate of success to increase as we continue to fine tune our protocol. One limit to successful expansion we have found is the starting number of T cells. In the case of TILs, the number of T cells we isolate critically affects potential expansion. Although we can expand down to 2,000 healthy donor T cells, this does not appear to be the case with tumor infiltrating T cells.

Once T cells have been expanded in vitro they can be co-cultured overnight with autologous APCs, either EBV transformed B cells or mDCs, which have been pulsed with tumor lysates generated by the Spellman group. T cells will then be examined by flow cytometry using previously established protocols in our lab. 6 hours prior to analysis a Golgi inhibitor, monensin, and various antibodies for activation markers such as, CD107a, CD107b, CD154, CD69, and CD137 will be added to the T cells. CD107a and CD107b will assess degranulation by CD8 T cells, while CD154 and CD137 represent CD4 activation markers, and CD69 is an early activation marker on both CD4 and CD8 T cells. By analysis of these markers using flow cytometry, we can identify cells that are reacting to the tumor lysate. These cells can then be single cell sorted using the CoH Flow Cytometry Core FACS Aria II by gating on these tumor reactive T cells. The cells will be single cell sorted into a 96 well plate where they will undergo a T cell expansion protocol.

Recently the Spellman group finished production of the tumor lysate from various cell lines and we are in the process of deciding how to mix them together and in what concentrations. Once we have identified the most functional APC conditions and verified our T cell clone expansion protocol, we will proceed with the entire protocol using enrolled patient samples.

Expanding isolated T cells as clones

Tumor lysate reactive T cells will be single cell sorted as described above into a 96 well plate. We will use a protocol based on CD3/CD28 stimulating antibody, feeder cells (both irradiated allogeneic PBMCs and irradiated JY cells, which is a transformed B cell line). As of yet we have not tested this protocol, but will do so soon. Single cell sorting on the FACS ARIA will be done by the CoH Flow Cytometry Core technicians, who are experienced with such tasks. However, all staff involved with this project on our team will also be trained for independent use of the FACS ARIA, in the event the Core staff is not available.

We plan on testing the combined APC antigen pulsing and antigen reactive T cell identification protocol by using healthy donor T cells, healthy donor derived APCs, and CMV peptide pools as antigen. Since most people have T cells reactive to CMV, this technique should serve as a nice positive control. We have recently purchased a CMV peptide pool from Miltenyi Biotec that is a mix of 15 peptides that elicit both CD4 and CD8 T cell responses.

Outline of the project plan for the next 12 months

- Finalize optimal EBV Transformation protocol of PBMCs
 - Determine if EBV Transformed B cells properly present antigen to both CD4 and CD8 cells
- Finalize the optimal monocyte-derived dendritic cell protocol.
 - Determine if CD14 isolated monocytes result in more generated DCs than by the adherence method of isolating monocytes
 - Determine the optimal culture factors for DC maturation
- Optimize APC-T cell cocultures
 - Determine the optimal DC to T cell ratio
- Optimize flow cytometry identification of tumor reactive T cells
- Optimize single-cell sorting and expansion of T cell clones protocol
- Once optimization is complete, begin using enrolled patient samples for progression through the entire protocol
 - Expanded T cell clones will be sent to the Denver team for further assays

Personnel

1. Peter P. Lee, MD – project PI (40% effort)
2. John Yim, MD – CoH Surgical Oncology (5% effort)
3. Joanne Mortimer, MD – CoH Medical Oncology (no salary requested on EHSA)
4. Jing Zhai, MD, PhD – CoH Pathology (no salary requested on EHSA)
5. Colt Egelston, PhD – post doc (100% effort)
6. Diana Simons – Research Associate II (95% effort)
7. Neta Zuckerman, PhD – post doc (40% effort)
8. Andrew Gewitz – Graduate Student (50% effort)

KEY RESEARCH ACCOMPLISHMENTS (over 6 months)

- Recruited an excellent team of 2 PhD post-doc, 1 graduate student, and 1 research associate II.
- Built strong collaborations with CoH clinical colleagues in surgery, medical oncology, and pathology to facilitate patient enrollment and samples acquisition.
- Human subjects protocol approved by CoH IRB and DoD HSRRB.
- Developed an efficient system of identifying, recruiting, and consenting subjects, and to obtain samples from the operating room to pathology and to our laboratory.
- Tested multiple protocols to maximize recovery of immune cells and tumor cells from tumor and lymph node specimens.
- Optimized T cell isolation and expansion protocols from primary tumor and TDLNs.

REPORTABLE OUTCOMES

On-going from efforts during this first year.

CONCLUSION:

Over the first 6 months of this award, we have focused on building a strong team, clinical collaborations, patient enrollment and samples acquisition. Our team has tested and optimized a number of protocols to process samples, generate antigen-presenting cells, and expand T cells for testing against tumor lysates from the Spellman group. This process is nearly complete, and we are positioned to work through the entire process as outlined in the SOW. We anticipate tumor-reactive T cell clones from patients will be generated starting in Year 2, which will lead downstream to antigen discovery.

REFERENCES:

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- O'Neill, D. W. and N. Bhardwaj (2005). "Differentiation of peripheral blood monocytes into dendritic cells." Curr Protoc Immunol **Chapter 22**: Unit 22F 24.

APPENDICES:
None at this time

SUPPORTING DATA:

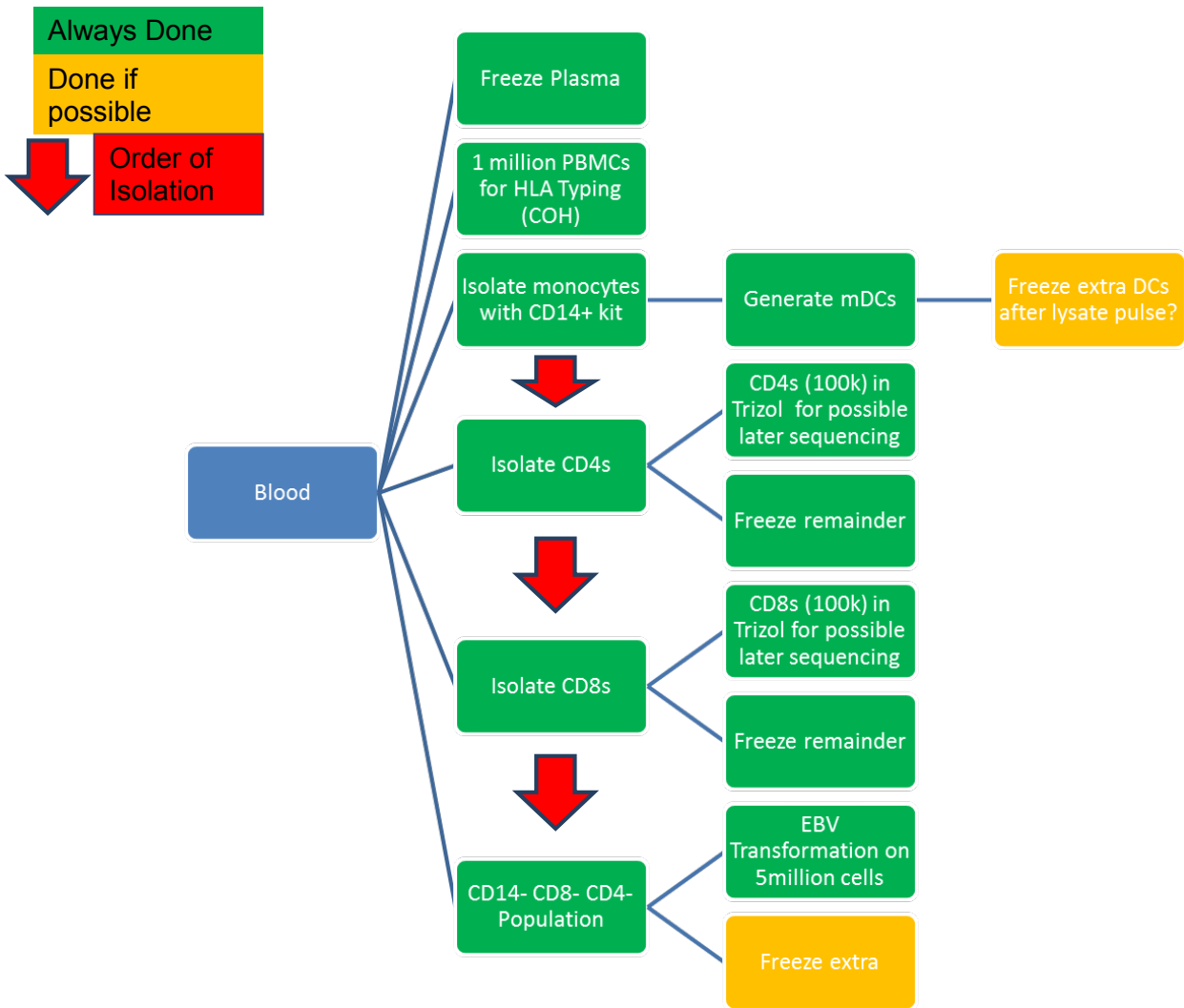


Figure 1. Flow of blood sample cells for various projects.



Figure 2. Flow of tumor sample cells between for various projects.

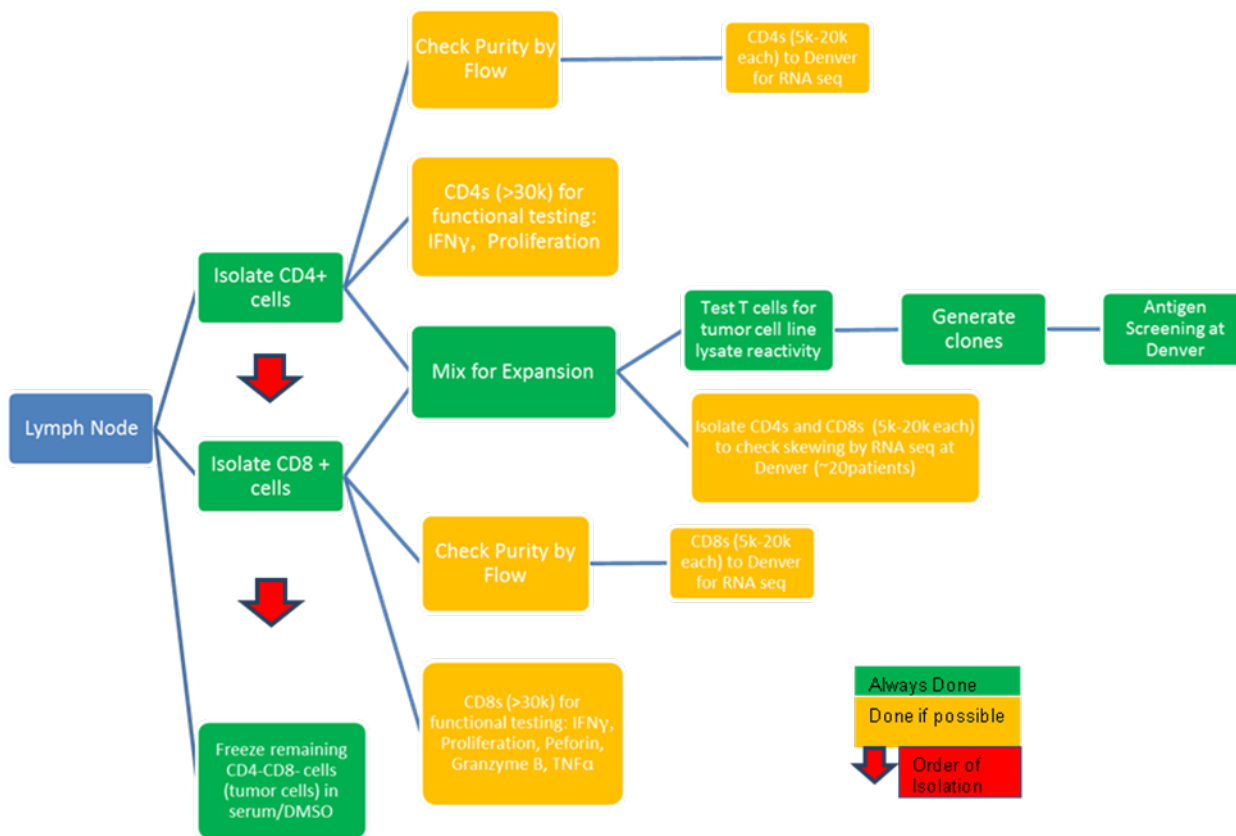


Figure 3. Flow of tumor sample cells between for various projects.

MONOCYTE DERIVED DCS



EBV TRANSFORMED B CELLS

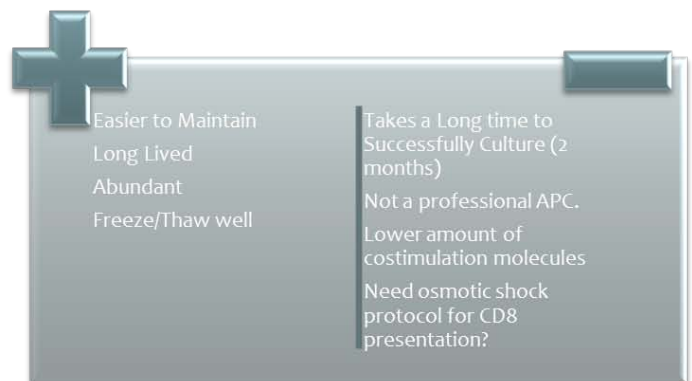


Figure 4. Comparison of monocyte derived dendritic cells to EBV Transformed B cells.

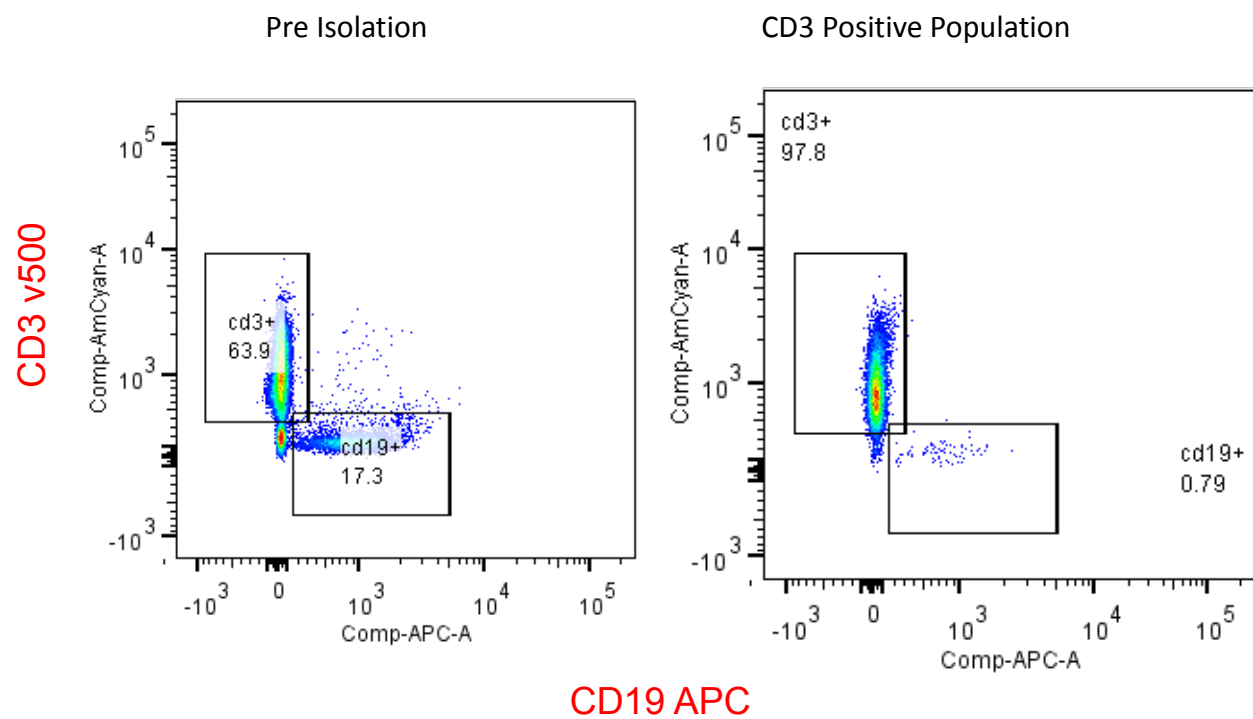


Figure 5. Trial isolation of T cells from healthy donor PBMCs using Dynall CD3 Flow Comp Kit.

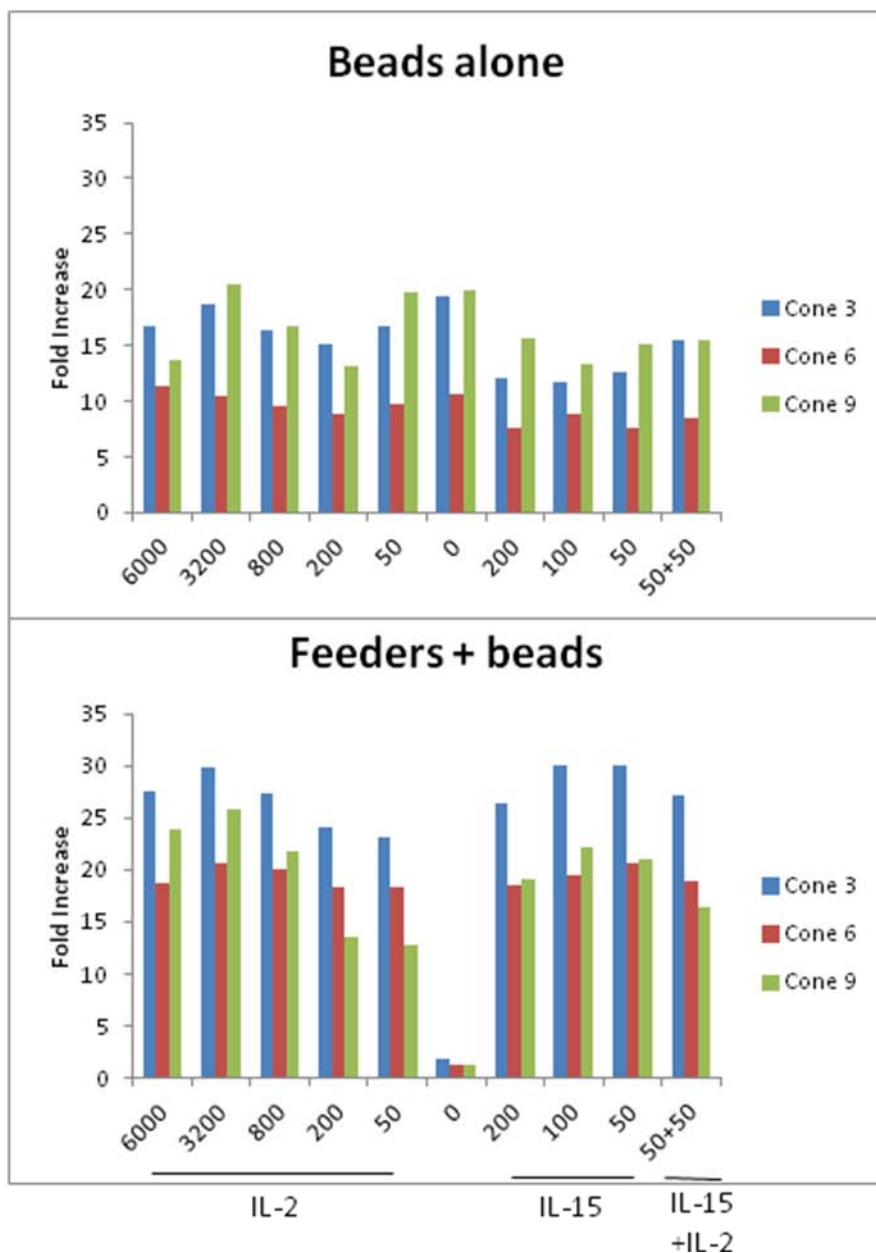


Figure 6. T cells were expanded with CD3/CD28 beads with either IL-2 (shown in IU/mL) or IL-15 (shown in ng/mL) at an initial dose on Day 0. For IL-2 cells were fed with half that initial dose on Day 3 and Day 5 and for IL-15 cells were fed with the same dose on Day 3 and Day 5. Here we show with or without feeder cells. Fold increase was measured by gating on CD8 cells with flow cytometry and calculating the fold increase in cells before and after the expansion protocol. Three separate ‘cones’, or healthy PBMCs are shown.

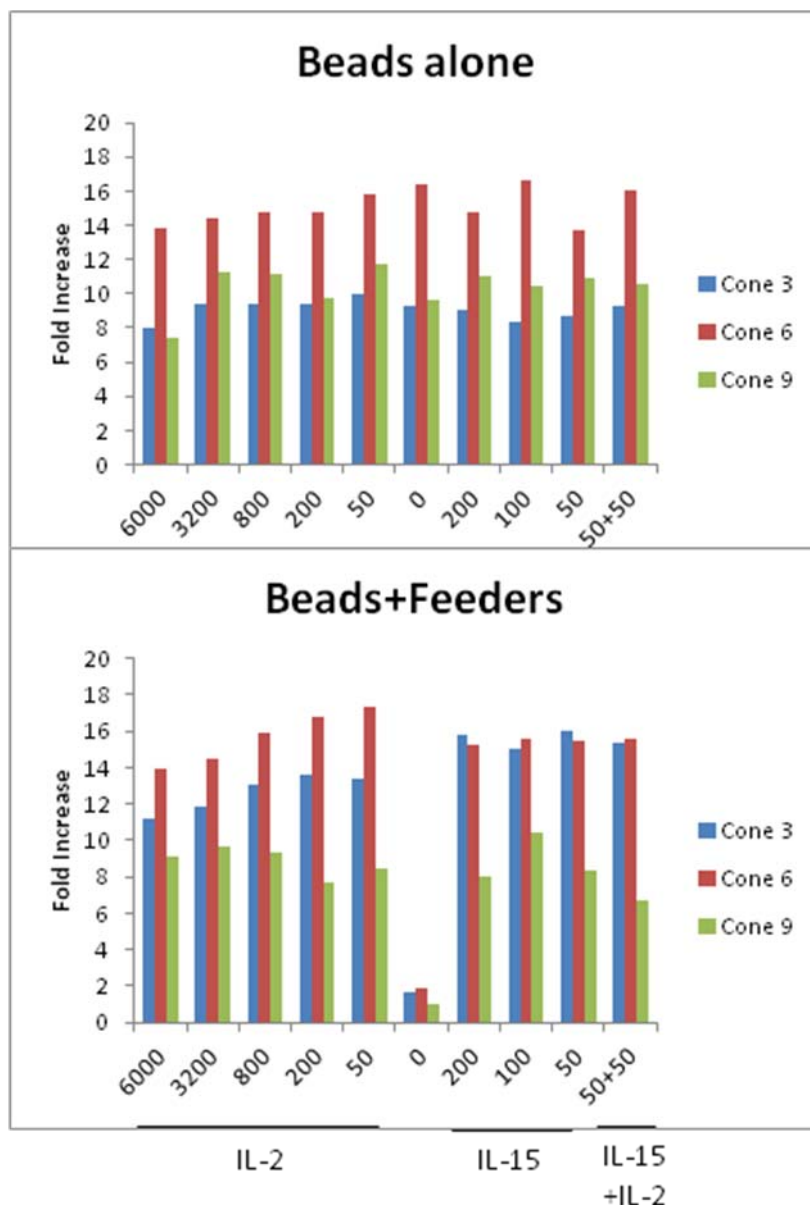


Figure 7. T cells were expanded with CD3/CD28 beads with either IL-2 (shown in IU/mL) or IL-15 (shown in ng/mL) at an initial dose on Day 0. For IL-2 cells were fed with half that initial dose on Day 3 and Day 5 and for IL-15 cells were fed with the same dose on Day 3 and Day 5. Here we show with or without feeder cells. Fold increase was measured by gating on CD4 cells with flow cytometry and calculating the fold increase in cells before and after the expansion protocol.